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Retargeted Clostridial Neurotoxins as Novel Agents for Treating Chronic Diseases

Felix L. Yeh,[†] Yiming Zhu,[‡] William H. Tepp,[§] Eric A. Johnson,[§] Paul J. Bertics,[‡] and Edwin R. Chapman*,[†]

[†]Department of Neuroscience, Howard Hughes Medical Institute, University of Wisconsin, Madison, Wisconsin 53706-1510, United States

[‡]Department of Biomolecular Chemistry, University of Wisconsin, Madison, Wisconsin 53706-1532, United States

[§]Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706, United States

Supporting Information

ABSTRACT: Botulinum neurotoxin (BoNT) A and B are used to treat neuropathic disorders; if retargeted, these agents could be used to treat medical conditions that involve secretion from nonneuronal cells. Here, we report novel strategies for successfully retargeting BoNTs, and also tetanus neurotoxin (TeNT), to primary human blood monocyte-derived macrophages where BoNT/B inhibited the release of tumor necrosis factor-α, a cytokine that plays a key role in inflammation. Furthermore, mice treated with retargeted BoNT/B exhibited a significant reduction in macrophage (MΦ) recruitment, indicating that these toxins can be used to treat chronic inflammation.

The clostridial neurotoxins (CNTs), which include seven BoNT serotypes (A−G) and tetanus neurotoxin (TeNT), have been integral for studying neuroexocytosis.[1] Intoxication by BoNTs causes flaccid paralysis, whereas TeNT causes rigid paralysis. Currently, most, if not all, therapeutic applications of the BoNTs involve the inhibition of the release of neurotransmitters from neurons.[2] The targets for these toxins are neuronal SNARE proteins. SNAREs, which include syntaxin and SNAP-25 on the target membrane and synaptobrevin on vesicles, form the core of a conserved membrane fusion machine that mediates neuronal exocytosis.[1,3]

However, it is well established that SNARE proteins are ubiquitously expressed and are important for secretion in many other cell types, which include immune cells, including eosinophils, neutrophils, MΦs, and mast cells.[4,5] Inhibition of secretion from these cells, using CNTs, could revolutionize treatment options for allergies (mast cells) and chronic inflammation (MΦs). However, the full therapeutic potential of these toxins has not been fulfilled because of the lack of toxin receptors on nonneuronal cells.

To target the toxins to nonneuronal cells, it was important to first investigate whether they were functionally active in the absence of their native receptors. HEK-293 cells were used as a model system to study alternative entry methods because of their lack of native toxin receptors. However, because most of the CNTs enter neurons via recycling synaptic vesicles,[6,7] it was important to retarget the toxins to an alternative vesicular compartment that is more commonly found in nonneuronal cell types.

Our strategy for redirecting the CNTs was to utilize the low-density lipoprotein receptor (LDLR), which is recycled through endosomes in virtually all cell types.[8] We transfected HEK-293 cells with an avidin-LDLR (AvLDLR) construct (Figure S1a of the Supporting Information) and then incubated the cells with biotinylated CNTs (Figure S1b of the Supporting Information). HEK-293 cells that were transfected with AvLDLR exhibited robust entry of several CNTs, as evidenced by significant cleavage of exogenously introduced SNAP-25 by biotinylated BoNT/A (biotin-BoNT/A) and biotin-BoNT/E (Figure S2a of the Supporting Information), and cleavage of transfected synaptobrevin II (syb II) by biotin-BoNT/B, biotin-BoNT/F, and biotin-TeNT (Figure S2b of the Supporting Information). Collectively, these results clearly demonstrate that the CNTs are active when retargeted to an alternative organelle lacking native toxin receptors, as suggested by a previous study.[9]

The fact that many primary cells are resistant to genetic modification[10] makes it difficult to introduce the CNTs via chimeric receptors. To overcome this obstacle, we used avidin to tether biotin-toxin to biotin-transferrin (Tr) or to a biotin-antibody directed against the extracellular domain of the LDLR to target recycling endosomes (Figure 3a of the Supporting Information). Indeed, when biotin-BoNT/A or biotin-BoNT/E was linked to the antibody or Tr through avidin, there was significant cleavage of SNAP-25 (Figure S3b,c of the Supporting Information). In addition, when biotin-BoNT/B, biotin-BoNT/F, or biotin-TeNT was attached to Tr, the toxins were able to enter cells and cleave their substrate, syb II (Figure S3d of the Supporting Information).

The next goal was to determine whether these toxins could be retargeted to MΦs, which are phagocytes that participate in various defense mechanisms in immune responses. They also play a maladaptive role in diseases, including sepsis, atherosclerosis, cancer, and chronic inflammatory disorders, including rheumatoid arthritis and Crohn's disease.[5]

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To retarget BoNT/B to RAW 264.7 cells, a mouse MΦ cell line, and cleave SNAREs, we modified the antibody-mediated delivery method and targeted the toxin to the Fc and complement receptor-mediated endocytosis pathway. Cellubrevin (ceb) levels in cells incubated with BoNT/B alone and BoNT/B with an anti-BoNT/A antibody (αA) were unaffected. With BoNT/B and αB (anti-BoNT/B), the toxin was able to enter cells and cleave ceb (Figure 1a). None of these treatments significantly affected cell viability (Figure 1b).

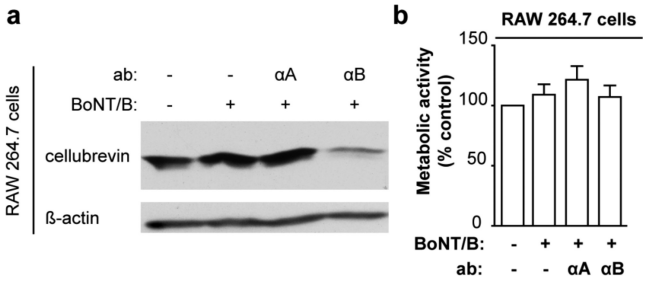

Figure 1. BoNT/B incubated with a serotype-specific antibody enters RAW 264.7 cells to cleave ceb. (a) BoNT/B (50 nM) and αB (combined at a 1:1 molar ratio) were combined and incubated with RAW 264.7 cells for 48 h, resulting in cleavage of 68% of ceb. ceb levels were unaffected in cells incubated with BoNT/B alone or BoNT/B incubated with αA. (b) MTS assay indicating no significant decrease in metabolic activity in response to toxin treatment vs the control. Error bars represent the standard error of the mean (SEM) (n = 3).

In addition to their ability to phagocytose and degrade microbes, MΦs also release a variety of cytokines that regulate inflammation and the recruitment of other immune cells. To determine the effect of SNARE cleavage by retargeted BoNT/B, we measured the release of cytokines and other factors from human BMDMs. Cells treated with BoNT/B, in conjunction with αB, exhibited an ∼50% decrease in interferon-γ and lipopolysaccharide (IFNγ/LPS)-induced TNFα release, without significantly affecting cell viability (Figure 2a and Figure S4a of the Supporting Information). We did not observe a reduction in the release of other cytokines and factors from these cells (Figure S5 of the Supporting Information).

The secretion of TNFα, a potent pro-inflammatory cytokine secreted primarily by MΦs and monocytes,[11] is thought to play a critical role in many diseases.[5,11−13] A dose−response study revealed that the IC_{50} of retargeted BoNT/B on TNFα release was ∼6 nM (Figure 2b). This effect was specific; the release of matrix metalloproteinase-9 (MMP-9), monitored as a control, was not reduced by the retargeted toxin, demonstrating that this enzyme is secreted through a distinct, BoNT/B-insensitive pathway (Figure S4b of the Supporting Information). Furthermore, the decrease in TNFα release was not associated with cytotoxicity (Figure S4c of the Supporting Information). We note that the increases in metabolic activity at higher doses of retargeted toxin might potentially explain the concomitant increase in MMP-9 release over this concentration range. The donor pool mainly consisted of two groups: clinically diagnosed allergic asthmatics and allergic patients without asthma. Human BMDMs from both groups exhibited significant decreases in TNFα release (Figure 2c,d).

The antibody-mediated delivery method illustrates the effectiveness in retargeting BoNT/B to human BMDMs; however, use of the intact toxin can potentially result in the

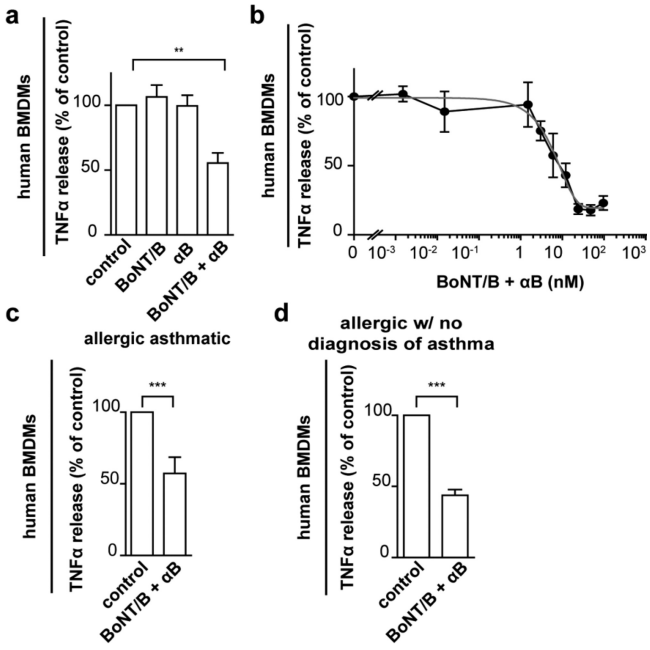

Figure 2. Retargeted BoNT/B reduces the secretion of TNFα from primary human BMDMs. (a) Release of TNFα from human BMDMs monitored by ELISA. When BoNT/B (6 nM) and αB (combined at a 1:1 molar ratio) were incubated with BMDMs for 48 h, the release of TNFα was reduced by 45%. The trial with antibody and BoNT/B alone was not significantly different from the control. Error bars represent SEM (n = 7; $p \leq 0.01$). (b) Dose response of retargeted BoNT/B on the release of TNFα from human BMDMs (fitted line colored gray). The concentrations indicated are for BoNT/B, which was preincubated with αB. Error bars represent SEM ($n \geq 3$; R^2 = 0.65; IC_{50} = 6.1 nM). (c and d) The release of TNFα from human BMDMs from allergic asthmatic patients (c) was reduced by 50%, while BMDMs from allergic patients with no diagnosis of asthma (d) exhibited a 39% reduction when incubated with 6 nM retargeted BoNT/B. Error bars represent SEM ($n \geq 5$; $p \leq 0.01$).

intoxication of neuronal cells. Therefore, further experiments were performed utilizing a truncated toxin, BoNT/BΔHC, which lacks the HC domain that mediates interactions with native receptors on neurons.[1]

To produce BoNT/BΔHC, we took advantage of a hypersensitive chymotrypsin site that lies between the translocation (HN) and HC domain of the protein (Figure S6a of the Supporting Information).[14] Toxin that was treated with chymotrypsin resulted in an ∼113 kDa fragment, which could be reduced by β-mercaptoethanol (BME) into two fragments corresponding to the HN domain and L chain (Figure S6b,c of the Supporting Information). To confirm the removal of the neurospecific binding domain, we tested whether BoNT/BΔHC could enter and intoxicate hippocampal neurons. As expected, BoNT/BΔHC had no detectable activity as demonstrated by the lack of syb II cleavage (Figure S6d of the Supporting Information). Thus, BoNT/BΔHC, preincubated with the antibody, is not targeted to neurons.

Next, primary human BMDMs treated with BoNT/BΔHC and αB led to a ∼25% decrease in TNFα release (Figure 3a), indicating that the HC domain is dispensable for targeted entry into human BMDMs. Finally, when BoNT/BΔHC and αB were administrated in mice with thioglycollate-induced inflammation, FACS (fluorescence-activated cell sorting) analysis revealed an ∼35% decrease in the extent of MΦ

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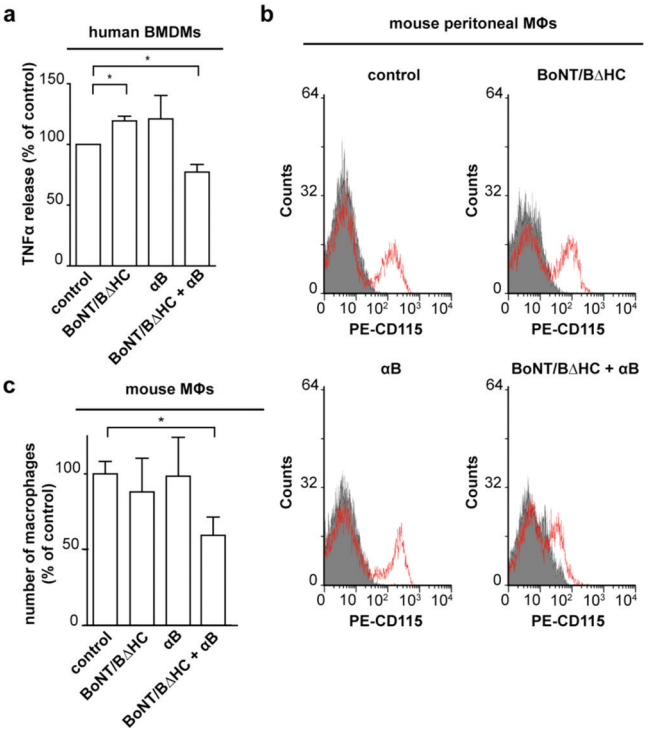

Figure 3. Retargeting of BoNT/BΔHC to MΦs. Injection into mice reduces the extent of MΦ recruitment in vivo. (a) The release of TNFα was assessed after treatment with BoNT/BΔHC (1 nM) and αB for 48 h and reduced by 23% vs the control. Error bars represent SEM (n = 3; $p \leq 0.05$). (b) FACS analysis of intraperitoneal cells, isolated from mice treated with the indicated agents, exhibited a significant decrease in the CD-115-positive MΦ population (red line) in the presence of BoNT/BΔHC and αB. Cells incubated with a control antibody are represented by the dark gray area. (c) Mice treated with a mixture of BoNT/BΔHC (25 ng) and αB (42 ng) exhibited an ~35% decrease in the extent of MΦ recruitment 4 h post-treatment, while neither protein alone exhibited a decrease vs the control. Error bars represent SEM ($n \geq 8$; $p \leq 0.05$).

recruitment versus the control, implicating an overall reduced state of inflammation (Figure 3b,c). These data further confirm that BoNT/BΔHC is effective at suppressing selective inflammatory responses both in vitro and in vivo.

To further investigate the effects of BoNT/B on MΦ recruitment, we investigated whether retargeted toxins affected integrin trafficking in RAW 264.7 cells. Adhesion through β1 integrins and activation of CD11b are thought to be critical for differentiation of monocytes to MΦs.[15,16] We observed no significant differences in adhesion between treated and untreated cells (Figure S7a of the Supporting Information) or in CD11b surface expression (Figure S7b of the Supporting Information). Thus, we believe the reduction in the level of intraperitoneal MΦs is largely due to the reduced extent of recruitment of monocytes and leukocytes because of decreased levels of TNFα.[17]

The CNTs are powerful therapeutic agents that reduce the symptoms of many profound diseases, including cervical dystonia and migraine headaches.[18] The findings reported here demonstrate that these toxins can be engineered to target cells other than neurons.[19] In particular, targeting the CNTs to human MΦs can help alleviate symptoms in many diseases such as rheumatoid arthritis, Crohn's disease, psoriasis, and refractory asthma.[13] Blocking the effects of TNFα has also been shown to inhibit tumor vascularization[12] and the progression of atherosclerosis.[11] With the added benefit of the prolonged effects of BoNT/B,[18] this strategy introduces a powerful new class of therapeutics.

■ ASSOCIATED CONTENT

Supporting Information

Detailed methods, Figures S1−S7, and supporting text and references. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (608) 263-1762. E-mail: chapman@wisc.edu.

Author Contributions

F.L.Y. and Y.Z. contributed equally to this work.

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